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Technical Advance

A Multiplex SNaPshot Assay as a Rapid Method for Detecting *KRAS* and *BRAF* Mutations in Advanced Colorectal Cancers

Sandrine Magnin,*† Erika Viel,‡ Alice Baraquin,* Severine Valmary-Degano,†§ Bernadette Kantelip,†§ Jean-Luc Pretet,*† Christiane Mougin,*† Marthe Bigand,§ Benoît Girardo,§ Christophe Borg,‡¶‖ and Christophe Ferrand*¶‖

From the Platform of Molecular Biology of Cancers, and the Services of Medical Oncology,‡ and Pathology and Cytology,§ University Hospital of Besançon, Besançon; the EA 3181, IFR 133,† and INSERM UMR 645, IFR 133,¶ University of Franche-Comte, Besançon; and the EFS Bourgogne/Franche-Comte,‖ Besançon, France*

The analysis of *KRAS* mutations has become a prerequisite for anti-epidermal growth factor receptor therapy in patients with metastatic colorectal cancers. *KRAS* mutations are associated with resistance to treatment by monoclonal antibodies such as cetuximab and panitumumab and thus are correlated with a shorter progression-free survival. *BRAF* mutations also may play a role in treatment decisions. The widespread use of these targeted therapies has generated the need to develop cost-effective methods for routine *KRAS* and *BRAF* analysis. The aim of this study was to compare a multiplex SNaPshot assay with DNA sequencing and high-resolution melting analysis for identifying *KRAS* codons 12 and 13 and *BRAF* codon 600 mutations. Thus 110 routinely formalin-fixed and paraffin-embedded tissue blocks were tested by each method. The SNaPshot analysis detected *KRAS* and *BRAF* codon 600 mutations in, respectively, 34.5% (n = 38) and 10% (n = 11) of these tissue blocks. These results were confirmed by direct DNA sequencing and by high-resolution melting analysis. The costs and time constraints of each detection method were compared at the same time. In conclusion, our newly designed multiplex SNaPshot assay is a fast, inexpensive, sensitive, and robust technique for molecular diagnostic practices and patient selection. *(J Mol Diagn 2011, 13:485–492; DOI: 10.1016/j.jmoldx.2011.05.010)*

Mitogen-activated protein kinase and phosphatidylinositol-3′-OH kinase signaling pathways form a network that participate in tumorigenesis. Activation by mutation of the different adaptors of this molecular network will deregulate proliferation, differentiation, and cell survival.[1] In colorectal cancers (CRCs), the *KRAS*, *BRAF,* and *PIK3CA* genes are mutated in approximately 30% to 50% of cases, leading to the activation of Ras/Raf/mitogen-activated protein kinase and phosphatidylinositol-3′-OH kinase signaling pathways.[2,3] Targeting epidermal growth factor receptor (EGFR) with monoclonal antibodies such as cetuximab and panitumumab has been shown to be an effective therapy for metastatic colorectal cancer as a single agent or in combination with chemotherapy. Activation of mutations in codons 12 and 13 of *KRAS* is associated with resistance to both cetuximab and panitumumab in patients with metastatic CRC.[4,5] On the basis of these results, the American Society of Clinical Oncology and the National Comprehensive Cancer Network both recommend *KRAS* mutation testing before prescribing EGFR antagonist therapy for patients with metastatic CRC and have stated that alternative therapies should be prescribed when mutations are detected.[4,6,7] Nevertheless, tumors with wild-type *KRAS* account for approximately 30% to 40% of resistant patients. Recent findings suggest that as with *KRAS*, mutation of the *BRAF* gene involving codon 600 is associated with a low overall response rate to cetuximab and panitumunab and a poor prognosis.[8,9] Today *KRAS* and *BRAF* status are considered in selected patients with metastatic CRC who are candidates for anti-EGFR therapy. Recent studies also have demonstrated that in addition to *KRAS* and *BRAF*,

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Address reprint requests to Christophe Ferrand, Ph.D., INSERM UMR645, EFS Bourgogne/Franche-Comte, 1 Bd Alexandre Fleming, F-25020 Besançon cedex, France. E-mail: christophe.ferrand@efs.sante.fr.

mutations in *PIK3CA* exon 20 were associated with a low response rate to EGFR inhibitors in patients with wild-type *KRAS* tumor status.[10,11] However, because of the low numbers of patients with *PIK3CA* exon 20 mutations in the series, the size of the cetuximab resistance effect is uncertain, and these data need to be confirmed.

In this study we developed and validated in routine diagnostics a sensitive SNaPshot test that allows for the rapid and simultaneous identification of common hot-spot mutations in *KRAS* and *BRAF* oncogenes in persons with advanced CRC. Various methods have been described for the detection of *KRAS* and *BRAF* mutations such as pyrosequencing,[12,13] real-time PCR,[14] array analysis,[15] amplification refractory mutation system (Scorpion assay)[16], high-resolution melting (HRM) analysis,[15–18] and Sanger sequencing of PCR products.[9,15–18] The disadvantage of DNA sequencing is its relatively high cost and limited sensitivity. HRM analysis is a recently developed molecular technique that is a cost-efficient, closed-tube system without any post-PCR processing and shows greater sensitivity and specificity than Sanger sequencing for *KRAS* and *BRAF* mutation detection.[17,19] A primer extension-based method, SNaPshot, has been described previously for *KRAS* codons 12 and 13 mutations.[20] This method may be adapted for the simultaneous analysis of up to 50 biallelic single nucleotide polymorphisms (SNPs) in a single reaction. We and others have developed SNaPshot assays that are able to detect *KRAS* and *BRAF* mutations simultaneously on routinely used formalin-fixed and paraffin-embedded (FFPE) blocks.[21–23] However, currently it is very difficult to tell which test is the most reliable. Moreover, tumor samples are very heterogeneous with regard to fixation, biopsy, surgical specimen, and neoadjuvant treatment, and one "best" method could depend on the type of sample.

We report herein our experiment (validated in every experiment performed during daily routine) in screening for *KRAS* codons 12 and 13 and *BRAF* codon 600 by SNaPshot, HRM analysis, and sequencing. Because it is essential to screen patient samples rapidly and at little expense, the cost-effectiveness and the total necessary time to acquire results were compared for each of the three methods used.

Materials and Methods

Samples and DNA Extraction

FFPE tissue samples were obtained from 110 consecutive patients with advanced CRC who were treated in the Cancer Institute of Franche-Comte, France. Informed consent was obtained from all patients. FFPE tissues containing at least 60% tumor cells were selected after microscopic examination by a pathologist. For 42 samples, ten 10-μm-thick serial sections were cut from paraffin blocks and placed in a microcentrifuge tube for DNA extraction. For the other 68 paraffin blocks that contained <60% of tumor cells, a coring procedure was used to enrich for tumor cell DNA. DNA extraction was performed with use of the QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France) according to the protocol for fixed tissues. DNA was eluted twice in a volume of 50 μL and then quantified spectrophotometrically at 260 nm with a Nanodrop ND-1000 (Labtech, Palaiseau, France). All DNA samples were tested for integrity and amplifiability using four pairs of primers for *Tbxas* exon 9, *Rag1* exon 2, *Plzf* exon 2, and *Af4* exon 21 amplification in a multiplex PCR to generate products with a length of 100, 200, 300, and 400 bp (BIOMED-2 control gene PCR method).[24] Because the quantity of DNA does not reflect the template quality, the multiplex PCR control was conducted on all extracted DNA. PCR was performed in a 25 μL reaction containing 1× Qiagen Multiplex PCR Master Mix (Qiagen), *Plzf* and *Af4* primers at 0.5 μmol/L final concentration, *Tbxas* and *Rag1* primers at a final concentration of 0.25 μmol/L, and at least 3 ng template DNA (2.5 μL volume of extracted DNA). The following protocol was used: 95°C for 10 minutes, 34 cycles of 95°C for 45 seconds, 60°C for 1 minute, 72°C for 1 minute 30 seconds, and finally 10 minutes at 72°C. PCR products were controlled by running 15 μL in 2% agarose–Tris-borate-EDTA gel.

Plasmid Standards

Plasmids containing each of the seven most frequent mutations of *KRAS* (p.G12V, p.G12A, p.G12D, p.G12S, p.G12C, p.G12R, and p.G13D) and the wild-type *KRAS* exon 2 were prepared. PCR amplification of the target genes was performed using genomic DNA extracted from tumor cells of FFPE tissue samples and that had been previously sequenced and shown to carry the *KRAS* and *BRAF* mutations. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and cloned into a pGEM-T Easy vector using a TA Cloning Kit (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions. Colonies containing recombinant plasmids were picked and sequenced to validate recombinant plasmids. Plasmids were quantified spectrophotometrically. A dilution series for each plasmid carrying the mutant allele was prepared to evaluate the sensitivity of the SNaPshot assays. The dilution series consisted of 0%, 1%, 5%, and 10% of mutant plasmids diluted into wild-type plasmid. Cloned DNA encoding each mutation analyzed in this work and required to control the SNaPshot multiplex assay will be available on request.

KRAS *Exon 2 and* BRAF *Exon 15 Multiplex PCR Amplification*

A multiplex PCR assay was designed to amplify fragments of *KRAS* exon 2 (200 bp) and *BRAF* exon 15 (250 bp). Primers for multiplex PCR amplification were as follows: *KRAS* exon 2, 5′-AAGGCCTGCTGAAAATGACTG-3′ (forward), 5′-CAAAGAATGGTCCTGCACCAG-3′ (reverse); *BRAF* exon 15, 5′-CATAATGCTTGCTCTGATAGGAAA-3′ (forward), 5′-TCAGCAGCATCTCAGGGCCAAA-3′ (reverse). Multiplex PCR was performed with the Qiagen Multiplex PCR Kit (Qiagen) in a total volume of 25 μL containing 1× Qiagen Multiplex PCR Master Mix (providing a final concentration of 3 mmol/L $MgCl_2$), 0.5

μmol/L of each primer, and DNA extracted from paraffin-embedded material (2.5 μL containing at least 3 ng of DNA) or 2.5 μL of sterile water as a negative control. PCR conditions were: 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 1 minute, 72°C for 1 minute, and finally, 10 minutes at 72°C. Multiplex PCR products were checked for quality and yield by running 18 μL in 2% agarose–Tris-borate-EDTA gel. In case no amplified band was detected, a second run of PCR was made to increase the detection threshold. Five microliters of primary amplification product was used as a template for the secondary amplification with the conditions previously described, except that the number of cycles was limited to 25. The secondary multiplex PCR assay was applied to the previous negative control to detect possible contaminant amplification. PCR products from the second amplification were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized on a UV light transilluminator. After purification using the gel extraction kit NucleoSpin Extract II (Macherey-Nagel, Hoerdt, France), PCR products were analyzed for the presence of *KRAS* codon 12 and codon 13 mutations and *BRAF* codon 600 mutational status. Separate isolation of both DNA fragments using agarose gel is not necessary.

SNaPshot Analysis

SNaPshot analysis was performed prospectively for diagnostic purposes with use of the ABI Prism SNaPshot Multiplex kit (Applied Biosystems, Courtaboeuf, France). Amplified *KRAS* exon 2 and *BRAF* exon 15 were analyzed for the presence of *KRAS* mutations at nucleotides c.34, c.35, c.37, and c.38 and for *BRAF* mutation at nucleotide c.1799 using five primers that contained an additional poly(dC) tail at their 5′ end, allowing for their simultaneous detection (Table 1). Primers used for *KRAS* codons 12 and 13 were previously described.[20] Reactions were performed in a final volume of 5 μL, containing 1.5 μL of purified multiplex PCR product (2 to 10 μg/μL), 2.5 μL of SNaPshot Ready Multiplex Ready Reaction Mix, 0.5 μL of probe equimolar mix (each probe at 2 μmol/L final), and 0.5 μL of double-distilled water. Multiplex single base extensions were carried out for 25 cycles according to the following program: 10 seconds at 96°C, 5 seconds at 50°C, and 30 seconds at 60°C. SNaPshot products were then treated at 37°C for 1 hour with 1 μL of shrimp alkaline phosphatase at 1 U/μL diluted in 2.5 μL of shrimp alkaline phosphatase buffer 10× and 11.5 μL of double-distilled water. After heat inactivation of shrimp alkaline phosphatase for 15 minutes at 75°C, 2 μL of the labeled products were mixed with 9.5 μL of HiDi formamide and 0.5 μL of Genescan-120LIZ size standard. They then were separated using a 25 minutes run on an ABI Prism 3130 DNA sequencer with POP-7 matrix and 14 seconds for injection. The analysis was performed using GeneMapper ID software version 3.2.1 (Applied Biosystems).

Sequencing Analysis

To confirm SNaPshot results, the purified multiplex PCR products were sequenced for the detection of *KRAS* mutations in exon 2. The sequencing reaction was performed using the Big Dye terminator V3.1 cycle sequencing kit (Applied Biosystems) and the same reverse primer used for the multiplex PCR. The reaction mix consisted of 1 μL of terminator premix 1×, 0.5 μL of sequencing buffer 5×, 1 μL of primer at 10 μmol/L, 2.5 μL of double-distilled water, and 5 μL of cleaned template (25 to 50 ng/μL) in a total volume of 10 μL. The reactions were run according to the following protocol: one cycle of 96°C for 1 minute; 15 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 1 minute 15 seconds; five cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 1 minute 30 seconds; five cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 2 minutes. After purification with a NucleoSEQ kit (Macherey-Nagel), samples were run on an ABI Prism 3130 DNA sequencer and analyzed using Sequencing Analysis software version 5.2 (Applied Biosystems).

HRM Analysis

An HRM assay also was performed to detect *KRAS* and *BRAF* mutations using the same DNA preparation as for the multiplex PCR. Real-time PCR and HRM analysis were carried out consecutively on a LightCycler 480 (Roche Diagnostics, Meylan, France). PCR for *KRAS* exon 2 was performed as previously reported.[25] For *BRAF*, primers were selected to flank the V600E mutation in exon 15 and were designed as follows: 5′-ATAGGTGATTTTGGTCTAGCTACAG-3′ (forward), 5′- AGTAACTCAGCAGCATCTCAGG-3′ (reverse). Each reaction mixture contained 10 μL of LightCycler 480 High Resolution Melting Master 2× (Roche Diagnostics), 3 mmol/L of

Table 1. SNaPshot Primers for the Detection of *KRAS* Codons 12, 13, and *BRAF* Codon 600 Mutations

Gene	Primer	Sequence	Size (bp)	Mutation	Amino acid change
KRAS codon 12	c.34	5′-AACTTGTGGTAGTTGGAGCT-3′	20	c.34G>A	p.G12S
				c.34G>T	p.G12C
				c.34G>C	p.G12R
	c.35	5′-C_{10}ACTTGTGGTAGTTGGAGCTG-3′	30	c.35G>A	p.G12D
				c.35G>T	p.G12V
				c.35G>C	p.G12A
KRAS codon 13	c.37	5′-C_{20}TTGTGGTAGTTGGAGCTGGT-3′	40	c.37G>T	p.G13R
				c.37G>C	p.G13C
	c.38	5′-C_{30}CTGTGGTAGTTGGAGCTGGTG-3′	50	c.38G>A	p.G13D
BRAF codon 600	c.1799	5′-N_{38}GGTGATTTTGGTCTAGCTACAG-3′	60	c.1799T>A	p.V600E

$MgCl_2$, 200 nmol/L of each primer, 0.5 U of Uracil-DNA Glycosylase, 1.1 μL of double-distilled water, and 5 μL (1 to 100 ng) of genomic DNA. Incubation of the reactions with uracil-DNA glycosylase for 10 minutes at 40°C before PCR prevents carryover contamination. PCR conditions were: 95°C for 10 minutes, followed by 50 cycles of 10 seconds at 95°C, a touchdown of 63°C to 58°C for 10 seconds (1°C/cycle), and 10 seconds at 72°C. After amplification, the PCR product was denaturated at 95°C for 1 minute and cooled down to 40°C for 1 minute to allow for heteroduplex formation. The final step was performed from 70°C to 95°C with an increase of 1°C per second with 25 acquisitions per degree. The HRM curve analysis was performed with use of Gene Scanning software (Roche Diagnostics). All samples were run in triplicate.

Statistical Analysis

Results of *KRAS* and *BRAF* mutational analyses were used as categorical variables (presence or absence of the mutation). The correlation of *KRAS* mutation frequency with sections of FFPE tissue punch cores was assessed by Fisher's exact test. Statistical significance was set at $P < 0.005$.

Results

DNA Fragmentation Analysis

For any PCR-based technique, template quality is probably the most important factor. Before the analysis of the *KRAS* and *BRAF* mutational status, DNA quality control was performed using a PCR for four different-sized fragments (100, 200, 300, and 400 bp in length) of reference genes. The FFPE samples produced variable numbers of bands reflecting variable DNA integrity. Among the 110 DNA samples obtained from FFPE samples, 72 produced bands of 100 to 400 bp and 15 allowed amplification of 100 to 300 bp. For nine samples, PCR products of 100 to 200 bp could be amplified, and finally, 14 samples allowed amplification only of the smallest sized control amplicon of 100 bp.

Multiplex PCR and SNaPshot Analysis

The first development made to the SNaPshot assay was to optimize a single-tube multiplex PCR able to amplify both targets of interest simultaneously from the FFPE samples in a routine diagnostic situation. Small PCR product sizes (200 bp for *KRAS* exon 2 and 250 bp for *BRAF* exon 15) were chosen to optimize amplification of partially degraded genomic DNA obtained from FFPE samples. The minimal amount of template to generate sufficient PCR products for SNaPshot or DNA sequencing was estimated at 3 ng and thus was not a limiting factor. To detect the *KRAS* 200 bp fragment and the *BRAF* 250 bp fragment, 32 of the 110 tumor samples (29%) with only 100 to 200 bp and very faint 200 to 300 bp signals after quality control PCR needed a second amplification of the first PCR product. To prevent the risk of nonspecific amplification, we limited the number of cycles in the second PCR assay (20 cycles), and negative controls from the first reaction underwent the second run. With use of these conditions, no contamination of the negative controls was observed.

PCR products were used as a template to perform a SNaPshot assay based on a single-base extension method and were able to detect simultaneously four different SNPs of *KRAS* exon 2 and the c.1799T>A *BRAF* exon 15 mutation (p.V600E). Somatic mutations of *KRAS* occurring at bases c.34, c.35, c.37, and c.38 from the start codon give rise to amino acid substitutions at codon 12 and 13. In this method, the primers described in Table 1 anneal one base 5′ of the locus that is to be typed. The DNA polymerase contained in the reaction mix appends a single fluorescently labeled dideoxynucleotide triphosphate to the 3′ end of the DNA primer. Detection of extended products was based on the four different fluorescently labeled dideoxynucleotide triphosphates and extension primers with oligonucleotide tails of differing lengths, thus controlling the concise length of the entire chromatogram to 60 bases. In our experiment, the difference in length between adjacent primers is 10 bp. Mutations are easily identified on the basis of peak size and color. When a mutation is present, an alternative dideoxynucleotide triphosphate is incorporated, resulting in a different colored peak. A mutant peak is considered positive if it is three times above background noise in the wild-type sample. The mobility of extended primers in capillary electrophoresis is determined by their size, nucleotide composition, and dye.

Validation and Sensitivity of the SNaPshot Assay

Validation was performed using primary tumor samples that had been previously tested by sequencing and shown to carry the mutations of interest. A representative example of common mutations detected is shown in Figure 1. In the wild-type sample electropherogram (Figure 1A), the four blue peaks indicate the four known SNPs of *KRAS* gene codons 12 and 13, and the red peak corresponds to the codon 600 SNP of the *BRAF* gene. In mutant samples, an additional peak overlapping or juxtaposing one peak of the wild-type electropherogram is indicative of the presence of a heterozygous mutation. In Figure 1B, two peaks at nucleotide c.34 showing "G" and "C" genotype indicate a c.34 G>C heterozygous mutation resulting in a p.G12R KRAS protein mutation. Figure 1, C–E, shows a c.35 G>A; p.G12D, a c.37G>T; p.G13C, and a c.38G>A; p.G13D *KRAS* mutation, respectively.

To evaluate the sensitivity of SNaPshot assays, DNA from mutant plasmids harboring mutations of interest was diluted into wild-type plasmid in proportions of 10%, 5%, and 1%. Our experiments on plasmid model systems indicated the possibility of identifying at least 5% of mutated alleles in a background of wild-type DNA. Examples of sensitivity data are illustrated in Supplemental Figures S1 to S7 (available at *http://jmd.amjpathol.org*).

Mutational Analysis of KRAS *and* BRAF *Genes in Clinical Samples*

We used multiplex PCR plus SNaPshot to test 110 FFPE colon tumor samples collected routinely. *KRAS* mutations were found in 38 (34.5%) of 110 tumor samples analyzed, and *BRAF* c.1799 T>A; p.V600E mutation was detected in 11 patients (10%). Of the 38 *KRAS* mutant cases, 32 cases (29%) of codon 12 mutation were found, including 15 cases (13.6%) of p.G12D, seven cases (6.4%) of p.G12V, five cases (4.5%) of p.G12A, four cases (3.6%) of p.G12C, and one case (0.9%) of p.G12S. Six cases (5.5%) of codon 13 mutation were found, as were all of the p.G13D mutant (Table 2). This finding is in line with the frequencies reported in the literature[2,3] and is consistent with previously published reports showing that *KRAS* codons 12 and 13 and *BRAF* codon 600 mutations are mutually exclusive.[8,19] The c.37G>T; p.G13C and c.37G>C; p.G13R mutations in CRCs are rare (0.8% and 0.3% respectively). Probably because of the low frequency, no mutation was detected by the *KRAS* c.37 assay in our cohort. Nevertheless, we validated before the c.37 mutation detection using a lung adenocarcinoma sample that had been previously tested and shown to carry the *KRAS* c.37G>T mutation (Figure 1). Tumor sample extraction from paraffin-embedded tissue is another variable parameter. We then attempted to find a correlation between the detection of mutations in the *KRAS* gene and specimens prepared either by sections or cores in paraffin blocks. *KRAS* was mutated in 25 (37%) of 68 coring samples and in 13 (31%) of 42 sections (Table 3). No significant correlation was noted between the frequency of *KRAS* mutation detection and the specimens preparation ($P = 0.42$, Fisher's exact test).

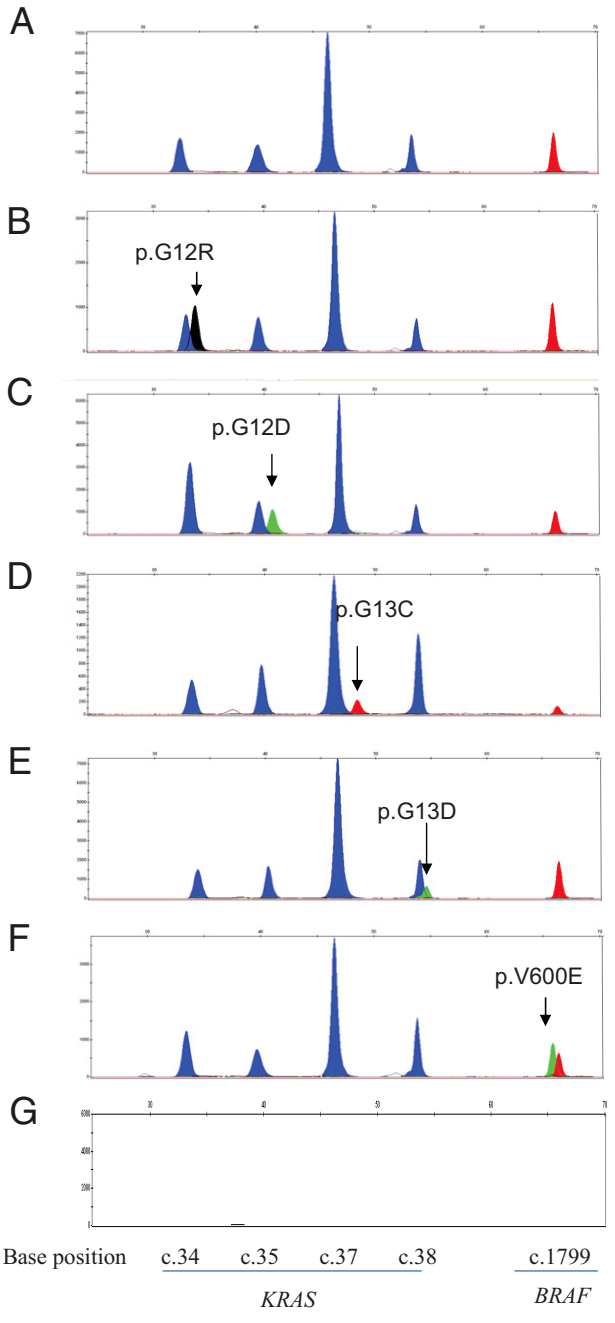

Figure 1. Assay validation of SNaPshot genotyping. Each peak corresponds to a specific extended primer. Positions of nucleotides are indicated at the **bottom** of the figure. **Arrows** indicate the location of each mutation. Bases are represented by the following colors: A = green; C = black; G = blue; T = red. **A–C**, **E**, and **F** show the results obtained using DNA extracted from FFPE colorectal tumor tissue. **D:** Genotyping result obtained using DNA extracted from lung adenocarcinoma. **G:** An example of no-DNA negative control. A wild-type case (**A**) and cases harboring a p.G12R (**B**), a p.G12D (**C**), a p.G13C (**D**), and a p.G13D (**E**) mutation of the *KRAS* gene and a mutated case (p.V600E) of the *BRAF* gene (**F**) are shown.

Table 2. Distribution of Detected *KRAS* Mutations as Determined by SNaPshot

Nucleotide change	Protein mutation	Number of samples	Relative %	Total %*
Codon 12				
c.35G>A	p.G12D	15	39.5	13.6
c.35G>T	p.G12V	7	18.4	6.4
c.35G>C	p.G12A	5	13.2	4.5
c.34G>T	p.G12C	4	10.5	3.6
c.34G>A	p.G12S	1	2.6	0.9
Codon 13				
c.38G>A	p.G13D	6	15.8	5.5
Total		38	100	34.5

*Of 110 cases.

Comparison of Molecular Testing Methods

We compared the multiplex SNaPshot assay with DNA sequencing and HRM analysis. All diluted plasmid mixtures used for SNaPshot sensitivity assessment were tested by HRM analysis and sequenced. Sequencing was sensitive to 5% to 10%, whereas HRM analysis read-

Table 3. *KRAS* Mutations Detection Frequency in DNA Extracted from Tissue Sections and Cores in Paraffin Blocks

DNA sequenced	Cores	Sections
KRAS mutation identified	25 (37%)	13 (31%)
Wild-type *KRAS*	43 (63%)	29 (69%)
Total	68	42

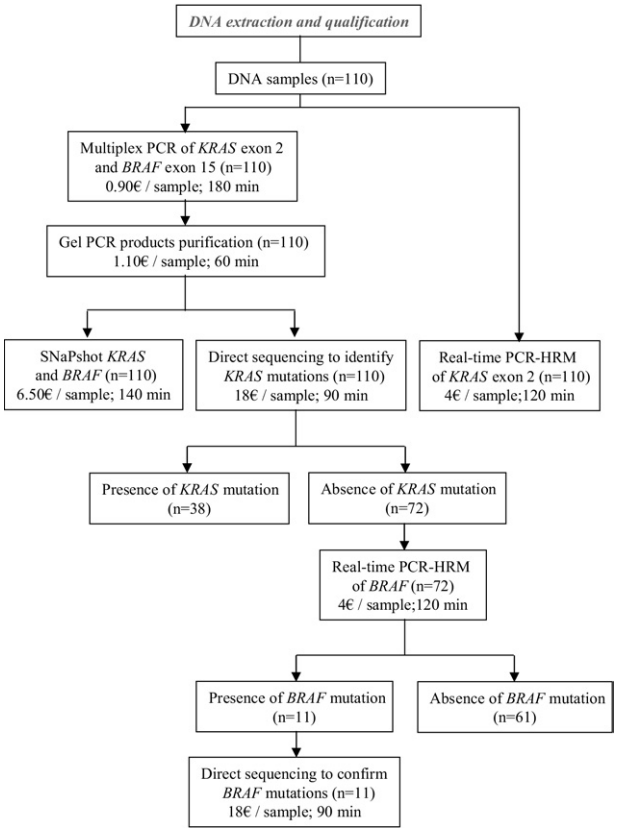

Figure 2. Strategies used to compare time and cost per sample for the three methods used to detect *KRAS* and *BRAF* mutations. Strategy 1: The 110 DNA samples were submitted to SNaPshot after *KRAS* exon 2 and *BRAF* exon 15 amplification detecting *KRAS* and *BRAF* mutations simultaneously. Strategy 2: Sequencing analysis was performed on the 110 cases for *KRAS* mutation identification using the same multiplex PCR products as for the SNaPshot strategy. Strategy 3: The 110 DNA samples were used for HRM *KRAS* exon 2 genotyping. Only samples without *KRAS* mutation were tested by HRM for *BRAF* mutation detection. Each *BRAF* mutation was confirmed by direct sequencing.

ily detected 5% mutant sequence (data not shown). We next compared the results obtained by SNaPshot with those obtained by BigDye Terminator sequencing on the 110 FFPE colon tumor samples (Figure 2). The mutational analyses of *KRAS* codons 12 and 13 were successfully performed in 100% of cases and confirmed using both methods. In HRM assays, all of the DNA samples were amplified and analyzed for *KRAS* exon 2 and *BRAF* exon 15. Because *KRAS* and *BRAF* mutations are predominantly mutually exclusive, with very few tumors (0.6%) containing a mutation in both genes,[9] it seemed reasonable to test for the presence of *BRAF* mutation by using the HRM assay only in wild-type *KRAS* tumors (72 samples) (Figure 2). All cases showing *KRAS* or *BRAF* mutations by SNaPshot were positive in HRM analysis, and no supplementary mutation was identified. HRM for *KRAS* mutations was performed using primers designed to span the entire exon 2 with a product size of 170 bp as reported.[25] Samples that had been previously tested and carried the *KRAS* mutations were included for comparison. Examples of melting curves and difference plots of the HRM data obtained from the FFPE colon tumor samples are shown in Figure 3A. For *BRAF* mutations analysis, a shorter 151 bp PCR amplicon with one single melting domain was designed to have a better resolution of genotypes and increase the sensitivity of mutation detection.[17] A wild-type control (DNA from the wild-type cell line) was used to normalize melting profiles of the other samples. The HT29 cell line, which carries a heterozygous c.1799T>A; p.V600E mutation, was used as a positive control (Figure 3B). Because any DNA alteration may produce an abnormal melting point curve, abnormal curves must be confirmed by an additional experiment, such as allele-specific competitive blocker PCR, direct sequencing, or pyrosequencing, which also show mutation identity.[25–27] In our experiment, each *BRAF* mutation was confirmed by direct sequencing.

Laboratory Working Time and Costs

The time and cost of each of the three methods used here for identifying *KRAS* codons 12 and 13 and *BRAF* codon 600 mutations (five SNPs) on each sample also were evaluated and reported in Figure 2. All procedures start with extracted DNA. Total time required was established at 6 hours 20 minutes for SNaPshot analysis versus 5 hours 30 minutes for automatic sequencing in an ABI Prism 3130 and 4 hours 10 minutes for HRM analysis in LightCycler480. When five SNPs are analyzed, SNaPshot using the ABI Prism SNaPshot Multiplex Kit costs 8.5€ (~$11.50 U.S.) per sample. This cost could be further reduced when more SNPs are simultaneously analyzed. In comparison, sequencing reaction using the Big Dye terminator V3.1 cycle sequencing kit costs 20€ (~$27 U.S.) per sample. DNA sequencing detection of *KRAS* and *BRAF* mutations involves *KRAS* exon 2 and *BRAF* exon 15 sequencing in two

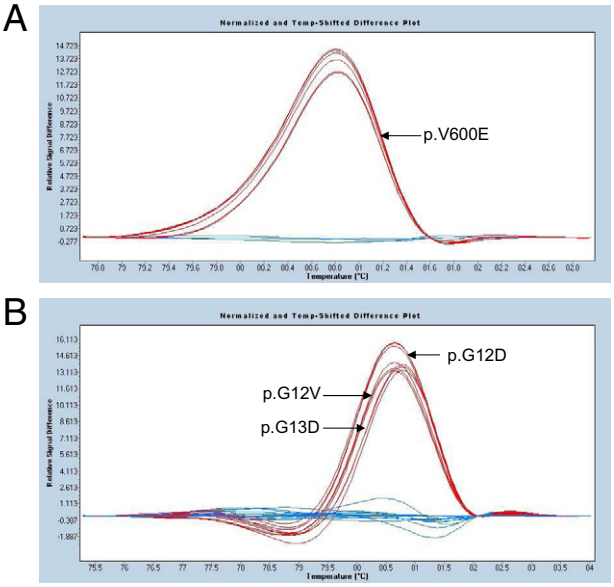

Figure 3. High-resolution melting analysis of *BRAF* exon 15 and *KRAS* exon 2. **A:** Difference plots of one colorectal cancer sample carrying the p.V600E mutation. *BRAF* mutated sample was normalized against wild-type DNA. Products of the positive control (HT29 cell line) and the mutated sample are shown in red curves. Products of wild-type templates are shown in blue. **B:** Difference plots showing the melting curve profiles of three cases revealing the presence of *KRAS* exon 2 mutation, namely p.G12D, p.G12V, and p.G13D. Mutated samples normalized against wild-type sample. Each sample was analyzed in triplicate.

separate reactions, which increases the cost per sample. The HRM methodology is inexpensive [8€ (~$11 U.S.)], but the need for confirmation with a second method, such as sequencing, increases turnaround time and cost.

Discussion

Because only the predictive values of *KRAS* codons 12 and 13 and *BRAF* codon 600 mutations have been confirmed clinically in patients with CRC treated with anti-EGFR antibodies,[11,25] we developed a SNaPshot assay as a diagnostically useful screening method for these common mutations in clinical FFPE specimens. The SNaPshot was validated in 110 routine heterogeneous tissue sets. Indeed, paraffin blocks are highly heterogeneous with respect to the quantity and distribution of tumor cells within the blocks, which is why a coring procedure was sometimes used to enrich for tumor DNA (always >60% of tumor cells), although no clear relationship was noted between the detection of mutation and the percentage of tumor sections.[16] According to pathology laboratory practices in the Franche-Comte region, particularly formalin fixation, the genomic DNA isolated from FFPE tissue is more or less fragmented, as DNA quality control PCR showed. Multiplex PCR developed before SNaPshot step amplifies small DNA fragments (200 bp for *KRAS* and 250 bp for *BRAF*). For poor-quality FFPE samples (with degraded or low-yield DNA), a second round of PCR was undertaken to amplify targets and increase the overall yield of the amplified fragments. This strategy allowed all of the 110 FFPE samples to be tested.

The multiplex SNaPshot assay described here is able to examine simultaneously four nucleotides in *KRAS* exon 2 and one nucleotide in *BRAF* exon 15 for 12 possible point mutations for *KRAS* and one for the *BRAF* gene. According to the Catalogue of Somatic Mutations in Cancer, for CRC, ~100% of the mutations in *BRAF* codon 600 are p.V600E. Typically the p.V600E BRAF protein mutation is a result of the T>A mutation at position 1799 of the *BRAF* nucleotide sequence. This p.V600E mutation also can result from the two-base mutation TG>AA at nucleotide positions 1799 to 1800. In this study, samples evaluated for activating mutations of *BRAF* underwent direct DNA sequencing. All p.V600E mutations resulted from a single bp change of T to A at nucleotide position 1799. Other mutations also can occur at *BRAF* codon 600, especially in melanoma.[18] These include p.V600K, p.V600D, and p.V600R. Here, the single base extension primer used for *BRAF* analysis at position 1799 also could detect a p.V600D mutation (c.1799_1800TG>AT). A confirmation of the p.V600 mutation is then necessary by using a reverse extension primer.

A variety of methods have been applied for somatic analysis of *KRAS* and *BRAF* mutations. Our results indicate that all methods used in this study—SNaPshot, direct sequencing, and HRM—yield similar results.

As with SNaPshot, direct Sanger sequencing is able to identify the specific mutation that may be present. However, to be successful, this methodology needs a sufficient amount of high-purity tumor material, probably because of the low sensitivity of the method previously reported.[26] Indeed, it is known that the quality of FFPE-derived DNA is not always good for PCR or sequencing reactions, and this was indeed the reason for the failed samples. We previously made this point with an assay performed in 160 tumor samples, which showed 35 mutations and 125 wild-type *KRAS*. In this previous cohort of 160 samples, SNaPshot analysis was able to identify *KRAS* mutations in 11 cases originally considered wild type, using direct sequencing analysis (data not shown). For this previous cohort, the percentage of tumor sections was not determined so far, and results were directly related to the poor DNA quality and to the reported difference in sensitivity between the two methods (~20% for dideoxy sequencing and 5% for SNaPshot).[23,26] In the assay reported here, the high concordance of results assessed by sequencing and SNaPshot most likely reflects the use of DNA extracted from samples consisting of at least 60% tumor cells and the improvement of fixation procedures.

Because a sensitive method based on HRM analysis to characterize *KRAS* and *BRAF* mutations has been described recently, we compared HRM with SNaPshot.[17,19] As published previously, HRM represents a more sensitive approach than direct DNA sequencing to detect somatic mutations in tumoral tissue with a sensitivity similar (5% to 6%) to that observed with SNaPshot[25] Moreover, HRM seems to be a suitable, fast, closed-tube methodology for testing FFPE samples.[16,17,19,28] This screening methodology also has the advantage of using a very low quantity of DNA. Our experiment revealed that about 1 ng of genomic DNA isolated from an FFPE sample is sufficient to obtain a result directly, whereas a second amplification sometimes is required to obtain a PCR product for a SNaPshot reaction. This observation is probably explained by the fact that the first step of the HRM assay is a simplex PCR reaction using LC Green for increasing the sensitivity of DNA detection. Moreover, HRM products (151 bp for *BRAF* exon 15 and 170 bp for *KRAS* exon 2) are shorter than SNaPshot multiplex PCR products (250 bp for *BRAF* and 200 bp for *KRAS*). However, despite these advantages, HRM analysis does not provide the identity of mutations that are potentially detected and required another method to confirm the mutation.

In conclusion, we have developed and validated a multiplex SNaPshot methodology for the robust and reliable genotyping of *KRAS* and *BRAF* mutations in a fast, simple, and cost-effective manner. Moreover, we provide a procedure for amplifying low-quality DNA (eg, DNA that is degraded or overfixed) by using two rounds of PCR and allowing for the analysis of *KRAS* and *BRAF* mutation in such samples. Because SNaPshot can analyze more than 10 SNPs at the same time in a single reaction, it is not a closed method. It therefore could allow for the simultaneous routine detection of common mutated oncogenes (ie, for *KRAS* codon 61) and thus the eligibility of patients with metastatic CRC for anti-EGFR therapy.

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